

Short Communication

Spectrofluorometric and high-performance liquid chromatographic determination of all-rac- α -tocopheryl acetate in virgin olive oilIl Kyu Cho^a, Jamil Rima^b, Chiou Ling Chang^c, Qing X. Li^{a,*}^aDepartment of Molecular Biosciences and Bioengineering, University of Hawaii at Manoa, 1955 East-West Road, Honolulu, HI 96822, USA^bLaboratory of Molecular Chemistry, Faculty of Science II, The Lebanese University, Fanar, P.O. Box 26110217, Lebanon^cUS Pacific Basin Agricultural Research Center, 2727 Woodlawn Drive, Honolulu, HI 96822, USA

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Abstract

A high-performance liquid chromatographic (HPLC) method was developed for the analysis of all-rac- α -tocopheryl acetate in olive oil. After extraction of all-rac- α -tocopheryl acetate from olive oil into hexane/ethyl acetate (4:1, v/v), it was measured with HPLC-diode array detection and regular external standard calibration and confirmed with liquid chromatography–mass spectrometry. Spectrofluorometric and standard addition methods were also used to quantify all-rac- α -tocopheryl acetate in olive oil. A standard addition method is based on fortification of known concentrations of all-rac- α -tocopheryl acetate in a sample. The concentrations ($30.4 \pm 1.2 \mu\text{g/mL}$) of all-rac- α -tocopheryl acetate determined with the HPLC method were comparable with those ($32.6 \pm 1.1 \mu\text{g/mL}$) with the spectrofluorometric method. Standards were checked for linearity giving correlation coefficients of higher than 0.999 in a concentration range from 0.5 to 20 $\mu\text{g/mL}$.

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1. Introduction

Vegetable oils, nuts, green leafy vegetables, and fortified cereals are common food sources of vitamin E (Rader et al., 1997). Antioxidants such as vitamin E act to protect cells against the effects of free radicals that are potentially harmful in energy metabolism, and they prevent oils from becoming rancid during storage and thus extend oil shelf life. Free radicals can damage cells and may contribute to the development of cardiovascular disease and cancer (Rader et al., 1997). Many research efforts have been made to determine whether vitamin E, through its ability to limit production of free radicals, might help prevent or delay the development of these chronic diseases (Blekas et al., 1995; Baldioli et al., 1996; Manzi et al., 1998). The acetate form of vitamin E is an ester that is more stable in the presence of light and oxygen than tocopherol. The shelf life of all-rac- α -tocopheryl acetate (vitamin E acetate) is greater than

that of the unesterified tocopherol and is excellent for skin care (Gensler et al., 1996). Studies have shown numerous skin benefits from the use of this vitamin (Gensler et al., 1996; Mayer et al., 1993; Alberts et al., 1996). All-rac- α -tocopheryl acetate is added to foods to increase vitamin E content, while α -tocopherol is a form of vitamin E in fruits.

Several high-performance liquid chromatographic (HPLC) studies for the determination of tocopherols and tocopheryl acetate in virgin olive oil and in soybean oil have been reported (Rader et al., 1997; Indyck, 1988; Dionisi et al., 1995; Gimeno et al., 2000a,b; Klejdus et al., 2004; Aoun et al., 2005; Tasioula-Margari and Okogeri, 2001). A comprehensive review on the HPLC analysis of α -tocopherol and related compounds in biological matrices has been recently published (Rupérez et al., 2001). Many methodological differences can influence the effectiveness of analysis. In general, separation of all tocopherol analogues is easier with a normal-phase HPLC system than a reversed-phase system (Mino et al., 1985; Rupérez et al., 2001; Sobczak et al., 1999; Kiyose et al., 2001; Ryyänen et al., 2004; Gimeno et al., 2000a,b; Huo

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et al., 1999). When only α -tocopherol is to be separated from other tocopherols and quantified, reversed-phase HPLC is often chosen (Rup  rez et al., 2001; Nilsson et al., 1978; Julianto et al., 1999). Methods for the analysis of tocopherol contents of erythrocytes with and without prior saponification are well described by Vatassery et al. (1993) and Gonzalez-Corbella et al. (1994), respectively. It is suggested that quantification of β -, γ -, and δ -tocopherols and tocopherolquinones could be achieved with satisfactory precision and accuracy only by use of a sensitive detector (Mino et al., 1985; Rup  rez et al., 2001; Sobczak et al., 1999; Vatassery et al., 1993). For this reason, fluorometric or electrochemical detection was preferred to UV (Sobczak et al., 1999; Hewavitharana et al., 2004).

The objectives of this study were to develop a spectrofluorometric method and an HPLC method for the analysis of α -tocopheryl acetate in olive oil without saponification, compare the two methods, and to show the potential of the spectrofluorometric method for the determination of all-rac- α -tocopheryl acetate in oils.

2. Materials and methods

2.1. Extraction of tocopherols

An aliquot of 10 mL of 50% aqueous ethanol was added in an olive oil sample (1 mL) in a 50-mL Pyrex glass tube with a Teflon screw cap. The olive oil sample was extracted three times with 10 mL of a mixture of *n*-hexane and ethyl acetate (4:1, v/v). The tubes were shaken at a speed of 500 strokes/min for 10 min. After being vortex-mixed and centrifuged (3000*g*, 5 min), the extracts were combined and dehydrated with a rotary evaporator at 40   C. The residue was dissolved in 2 mL of *n*-hexane, and was filtered through a 0.45 mm nylon filter prior to HPLC. An amount of 1 mL of olive oil was mixed with 9 mL of a mixture of ACN/hexane (4:1, v/v), and then an aliquot of 4 mL of the solution was placed directly in a cell for spectrofluorometric measurement.

2.2. Chemicals and reagents

All chemicals and solvents used were of analytical reagent grade or of the highest purity available. These reagents included HPLC-grade acetonitrile, ethanol, methanol, and hexane (Fisher ChemiAlert), ethyl acetate, diethyl ether, and acetonitrile (Aldrich), sodium hydroxide and sodium sulphate (Merck), all-rac- α -tocopheryl acetate (ICN Biomedical Inc.), and all-rac- α -tocopherol (Sigma). Ultra-pure water was prepared using a multi-Q filter system (Millipore). Olive oil was produced from Italian-grown olives (extra virgin olive oil).

2.3. Equipment

2.3.1. High-performance liquid chromatography

A Dionex BioLC system was used, consisting of a PDA-100 photodiode array (PDA) detector, AS50 autosampler,

GP50 gradient pump and column oven (Timberline 101) and controlled by Chromeleon software. The instrument was run at the following conditions: flow rate, 2.0 mL/min; column temperature, 30   C; detection wavelength, 280 nm; column, Inertsil ODS-2, 5 mm, 4.6    250 mm²; mobile phase, acetonitrile (ACN)/methanol (95%/5%, v/v); and injection volume, 20   L. Retention time of all-rac- α -tocopheryl acetate was 9.87 min.

2.3.2. Spectrofluorometric luminescence

A PTI Spectrofluorometry (Photo Technology International) was run at an excitation wavelength (λ_{ex}) of 295 nm and an emission wavelength (λ_{em}) of 325 nm for all-rac- α -tocopheryl acetate. A standard addition method (Muel and Lacroix, 1960; Rima et al., 1982) was used to calibrate all-rac- α -tocopheryl acetate contents in olive oils. The standard was all-rac- α -tocopheryl acetate added in olive oil.

2.3.3. Liquid chromatography–mass spectrometry

An Agilent 1100 series liquid chromatograph–mass spectrometer (LC–MS) was used, consisting of a vacuum degasser, binary pump, auto-sampler, column thermostat, diode array detector, and a 1100 series mass selected detector (MSD) controlled by Agilent ChemStation. Samples were analysed on Agilent 1100 series LC–MSD with atmospheric pressure chemical ionization (APCI) mode. The corona discharge electrode was set to 2.0 kV and the APCI probe temperature to 450   C. The APCI heater gas (nitrogen) was set to 400 L/h, and the nebulizer gas (nitrogen) at 80 p.s.i. Mass spectra were recorded at a scan range of 100–800 *m/z* and the protonated molecular ions ($[M + H]^+$) were measured (Vaule et al., 2004). The $[M + H]^+$ ions of all-rac- α -tocopheryl acetate and all-rac- α -tocopherol were 473 and 431, respectively.

LC-ion trap MS analyses were carried out on an UltiMateTM plus nano LC system interfaced to an Esquire HCT ion trap mass spectrometer (LC/ITMS) (Bruker Daltonics, Bremen, Germany). The gradient elution was started at 2% ACN and 0.1% formic acid in water for 5 min, ramped to 60% ACN in 10 min, and finally to 80% ACN for another 15 min. Flow rate was 150   L/min. An aliquot (5   L) of the sample solution (in a 20   L sample loop) was injected with an auto-sampler onto a C-18 nano-column (PepMap 100, 75   m i.d.    15 cm), which was connected to an ion source chamber (orthogonal) with a sheath gas flow at 3 units for MS analysis and adjusted to Expert mode (–2000 V capillary voltage of ion source, 200 ms max. accumulation time, 3.0 L/min dry gas, 150   C dry temp). Mass spectra were recorded at a scan range of 100–1000 *m/z*.

2.4. Calibration curve and recovery

The stock standard solution (100   g/mL) was prepared with 10 mg of all-rac- α -tocopheryl acetate or all-rac- α -tocopherol in 100 mL of ACN/hexane (4:1, v/v). The

working solutions for standard curves were prepared by diluting 0.5, 1, 5, 10, and 20 mL into 100 mL of ACN/hexane (4:1, v/v). Linearity and detection limits were established with working standard solutions at 0.5, 1, 5, 10, and 20 $\mu\text{g/mL}$ of all-rac- α -tocopheryl acetate. Recoveries (R%) were carried out with samples fortified with 30 $\mu\text{g/mL}$ and calculated as follows:

$$R\% = [(C_s - C_p)/C_a]100,$$

C_s is the concentration of sum of all-rac- α -tocopheryl acetate in the added samples, C_p the concentration of all-rac- α -tocopheryl acetate in the samples, C_a the concentration of all-rac- α -tocopheryl acetate standard added.

3. Results and discussion

3.1. Identification of all-rac- α -tocopheryl acetate

All-rac- α -tocopheryl acetate in olive oils was directly identified and quantified without saponification. Figs. 1A and B show HPLC–PDA chromatograms of all-rac- α -tocopheryl acetate in a standard solution and an olive oil extract, respectively. After optimization of HPLC conditions, a peak of all-rac- α -tocopheryl acetate was observed at 9.87 min. Identification of all-rac- α -tocopheryl acetate in

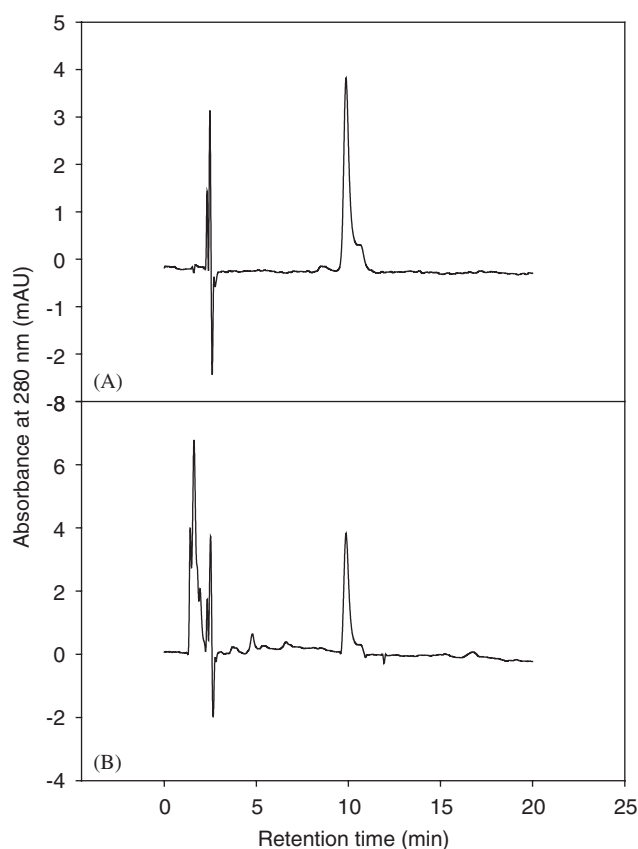


Fig. 1. HPLC–PDA chromatograms for determination of all-rac- α -tocopheryl acetate in olive oil. (A) 20 $\mu\text{g/mL}$ of all-rac- α -tocopheryl acetate in ACN/hexane (4:1, v/v); (B) sample extract of olive oil in n-hexane/ethyl acetate (4:1, v/v).

olive oil was confirmed with LC/ITMS (Fig. 2). Retention time of all-rac- α -tocopheryl acetate was 11.5 ± 0.2 min on the UltiMateTM plus nano LC system. The m/z value for all-rac- α -tocopheryl acetate was 473.6. The 601.7 m/z value in the olive oil extract was probably the tri-acetate form of all-rac- α -tocopheryl acetate [$472.8 + (43 \times 3) = 601.8$].

The m/z values of protonated molecular ions for all-rac- α -tocopheryl acetate and α -tocopherol were 473.3 and 431.3, respectively (not shown) when the mass spectra were obtained with LC–MSD in APCI mode. The presence of all-rac- α -tocopheryl acetate was also analysed with a spectrofluorometer. Figs. 3A and B show fluorescence

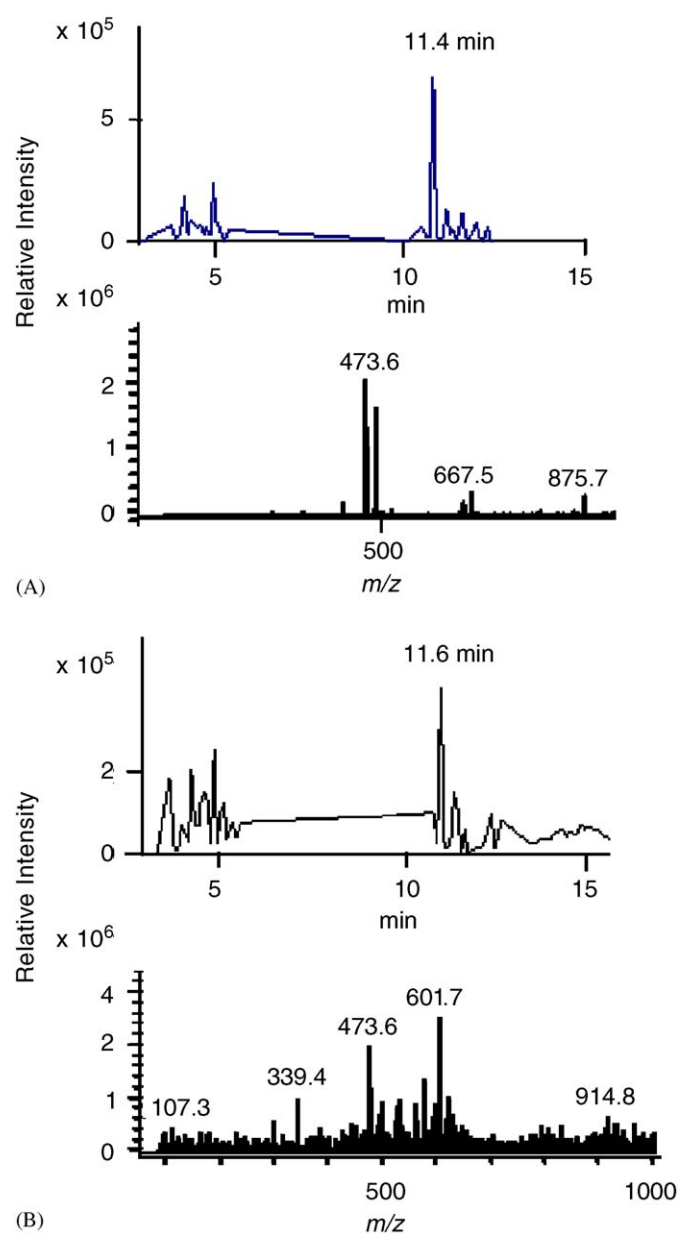


Fig. 2. LC/ITMS chromatograms and mass spectra of all-rac- α -tocopheryl acetate in standard solution (A) and olive oil extract (B). The top mass spectrum is the MS of the 11.4-min peak. The bottom mass spectrum is the MS of the 11.6-min peak.

spectra of all-rac- α -tocopheryl acetate in a standard solution (A) and olive oil extract (B), respectively.

3.2. Chromatographic method for quantification of all-rac- α -tocopheryl acetate

HPLC and spectrofluorometric methods for determination of all-rac- α -tocopheryl acetate in olive oil were validated for the selectivity, linearity and calibration range, accuracy, recovery, and limits of detection (Table 1). Identification was achieved by comparing the retention times of the peaks observed and mass spectra. HPLC standard calibration curves were constructed by spiking olive oil extracts with known amounts of all-rac- α -tocopheryl acetate in a concentration range of 1–20 $\mu\text{g/mL}$. The least-square method was used to calculate the regression equation. A strong linearity obtained indicates

that the HPLC method is appropriate for the quantification of all-rac- α -tocopheryl acetate. Correlation coefficients were higher than 0.99 in a concentration range 1–20 $\mu\text{g/mL}$ (Table 1). The method precision was evaluated with relative standard deviations (RSDs) of all-rac- α -tocopheryl acetate determination in five samples, which was 3.9% for HPLC–PDA. The average content of all-rac- α -tocopheryl acetate determined with HPLC–PDA was $30.4 \pm 1.2 \mu\text{g/mL}$ ($n = 5$) and the limit of detection by HPLC–PDA was 0.5 $\mu\text{g/mL}$ of all-rac- α -tocopheryl acetate (Table 1). The average recovery of five olive oil samples spiked with all-rac- α -tocopheryl acetate (30 $\mu\text{g/mL}$) determined with HPLC–PDA was $95.5\% \pm 2$. The detector response of HPLC–PDA was linear in the tested range of 20–400 ng/injection ($R^2 = 0.9867$) (Fig. 4A). Use of HPLC fluorescence detection may increase the method sensitivity and specificity.

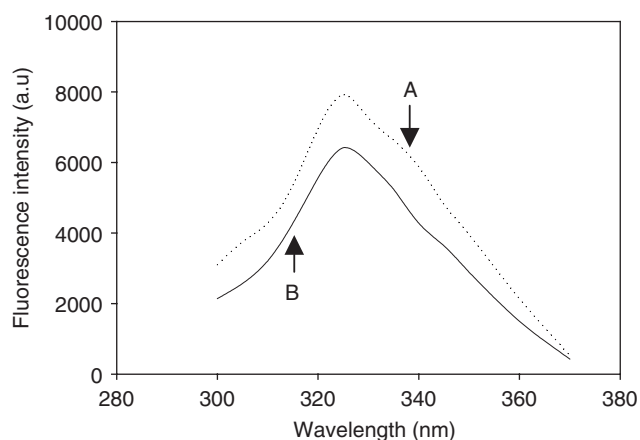


Fig. 3. Fluorescence spectra of all-rac- α -tocopheryl acetate standard (A) in ACN/hexane (4:1, v/v) solution; (B) in olive oil extract of ACN/hexane (4:1, v/v).

Table 1
Method validation parameters for determination of all-rac- α -tocopheryl acetate in virgin olive oil

Method	Spectrofluorometry	HPLC–PDA
<i>Standard linearity</i>		
Intercept	−2.467	−3.275
Slope	1.150	0.350
R	0.999	0.993
Range ($\mu\text{g/mL}$)	0.5–20	1–20
<i>Precision ($n = 5$)</i>		
Mean ($\mu\text{g/mL}$)	32.6	30.4
Relative standard deviation (RSD) (%)	3.5	3.9
<i>Accuracy</i>		
Mean recovery (%) \pm standard deviation	95.0 ± 5	95.5 ± 2
Detection limit ($\mu\text{g/mL}$)	0.01	0.5

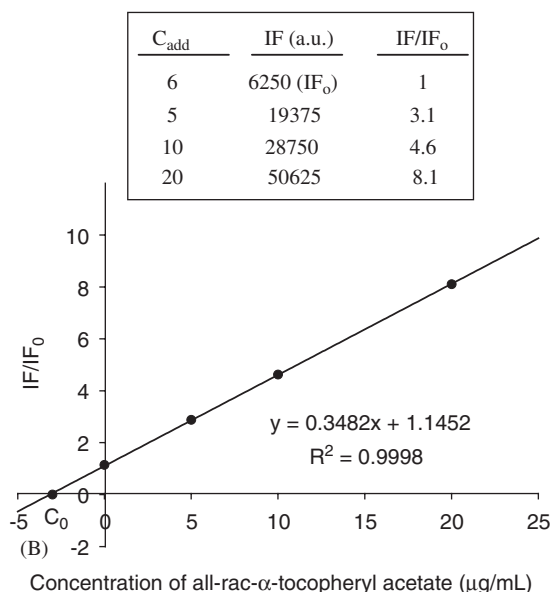
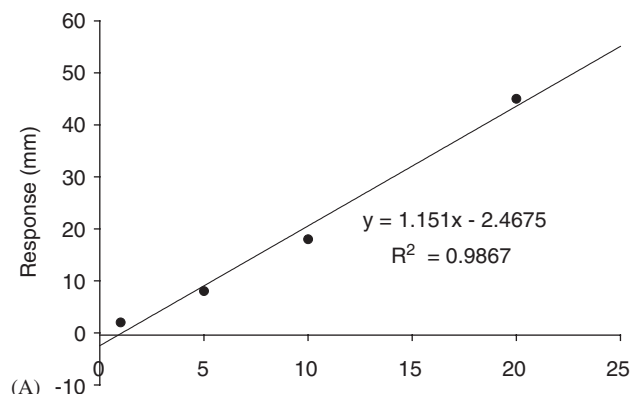


Fig. 4. (A) HPLC standard calibration curve of all-rac- α -tocopheryl acetate; (B) spectrofluorometric calibration curve of all-rac- α -tocopheryl acetate added in an olive oil sample at varying concentrations (IF₀ = fluorescence intensity before the standard addition, IF = fluorescence intensity after the standard addition; the inset for (B) shows the calculations).

3.3. Spectrofluorometric method for quantification of all-rac- α -tocopheryl acetate

A spectrofluorometric method was performed to analyse all-rac- α -tocopheryl acetate. A calibration curve follows an equation $IF^* = AC + B$, which is also $IF^* = (IF_0^*/C_0) \times C_{add} + IF_0^*$, where IF^* , normalized fluorescence intensity (arbitrary values), is equal to the ratio (IF/IF_0) of the fluorescence intensity after the standard addition (IF) and the fluorescence intensity before the standard addition (IF_0). C_0 is the solute concentration to be estimated. IF_0^* is the normalized fluorescence intensity of the starting solution (i.e., $IF^* = 1$ or $IF = IF_0$ when $C_{add} = 0$). C_{add} is the known added concentration. The concentration C_0 is determined by the negative intercept of the extrapolated line with the x -axis (Muel and Lacroix, 1960; Rima et al., 1982). It is noted that this method requires a calibration curve for each sample. The plot for IF^* vs. C_{add} is shown in Fig. 4B. The spectrum between 300 and 400 nm was recorded [an excitation wavelength (λ_{ex}) of 295 nm and an emission wavelength (λ_{em}) of 325 nm for all-rac- α -tocopheryl acetate]. The RSD of all-rac- α -tocopheryl acetate concentrations in five samples by the spectrofluorometric method was 3.5%. The average recovery of all-rac- α -tocopheryl acetate spiked in olive oil samples (20 μ g/mL) was $95\% \pm 5$. The average content of all-rac- α -tocopheryl acetate determined with the spectrofluorometric method was 32.6 ± 1.14 μ g/mL ($n = 5$). The limit of detection of the spectrofluorometric method was 0.01 μ g/mL (Table 1) as defined by a signal-to-noise ration of 3:1 (MacDougall and Crummett, 1980).

4. Conclusion

In summary, the spectrofluorometric and HPLC–PDA methods are sensitive and simple for the analysis of all-rac- α -tocopheryl acetate in olive oil. The spectrofluorometric method has advantages over HPLC, due to being fast, simple, and economical. However, the HPLC method has the advantages of separation of all-rac- α -tocopheryl acetate from interferences and accurate measurements. All-rac- α -tocopheryl acetate in olive oil can be confirmed with LC–MS.

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